

## SHORT REPORTS

Association of idiopathic venous thromboembolism with single point-mutation at Arg<sup>506</sup> of factor V

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Abnormal coagulation factor V may underlie the thrombotic events associated with resistance to activated protein C (APC). We analysed 27 consecutive patients with documented idiopathic (recurrent) thromboembolism for the occurrence of point mutations within the APC sensitive regions of blood coagulation factor V. In 10 patients we observed a single basepair mutation resulting in a substitution of Arg<sup>506</sup> to Gln. This mutation was significantly linked to in-vitro resistance to APC in these subjects. This mutation at Arg<sup>506</sup> of factor V may form the molecular basis for the thrombotic events associated with APC resistance.

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Venous thromboembolism has been associated with molecular defects in several haemostatic components: antithrombin III, protein C, protein S, plasminogen, and fibrinogen.<sup>1</sup> However, in over 90% of patients the cause remains obscure.<sup>2</sup> A poor anticoagulant response to activated protein C (APC) has been observed in about 20-30% of patients with an idiopathic predisposition to thromboembolic disease.<sup>3,6</sup> This abnormal response has been linked to a plasma factor which appeared to be identical to coagulation factor V.<sup>7</sup> These observations suggest that a molecular abnormality in factor V underlies the thrombotic events that are associated with a defective anticoagulant response to APC in vitro. We report linkage between resistance to APC and a single point-mutation at a putative APC cleavage site at Arg<sup>506</sup> of factor V.

We investigated 27 consecutive patients (13 men; mean age 53, range 23-79) with (recurrent) idiopathic episodes of thromboembolism confirmed by contrast venography, pulmonary angiography, or both. None of the patients received oral anticoagulants at the time of the study. Patients with cancer or lupus anticoagulants were excluded. No patient had an acquired or inherited deficiency of antithrombin III, protein C, protein S, or plasminogen. Routine screening of blood coagulation and fibrinolysis revealed no abnormality. Resistance to APC was assessed by the APC-dependent prolongation of the activated partial thromboplastin time (Coatest APC Resistance, Chromogenix, Sweden).<sup>3,5</sup> An APC sensitivity ratio  $\leq 2.0$  was considered to represent a defect in the anticoagulant response to APC.

Patients were analysed for the presence of mutations at Arg<sup>506</sup> in factor V with the following oligonucleotide primers: 5'-CATCAGT'TTCACCTCATCAGG3' (primer 506-2, nucleotides 1708-1730 of human factor V) and 5'-ATCAGAGCAGT'TCAACCAGGG3' (506-5, nucleotides 1414-1435). RNA was isolated from peripheral blood lymphocytes by the RNAzol B method (WAK Chemie, Bad Homburg, Germany) and cDNA was prepared.<sup>8</sup> Amplification by polymerase chain reaction with primers 506-2 and 506-5 yields a fragment of 316 basepairs, which encodes the part of factor V that contains

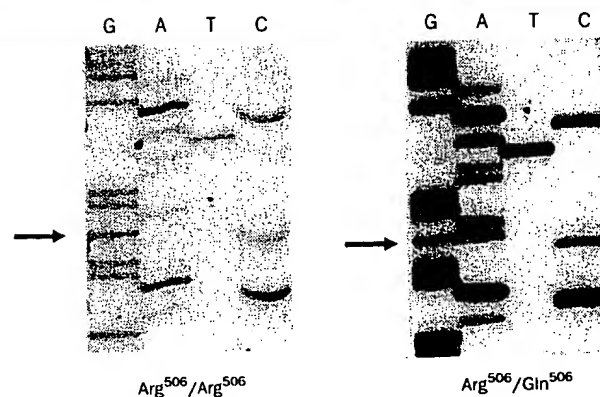


Figure 1: Sequence analysis of factor V cDNA

Factor V cDNA derived from patient heterozygous for Arg<sup>506</sup> to Gln mutation is shown in right panel. Heterozygosity is scored by occurrence of both a "G" and an "A" at second basepair of codon Arg<sup>506</sup> (CGA/CAA) of factor V (arrow). In left panel, sequence analysis of patient who does not carry the mutation is displayed. Arrow = single "G" observed at second basepair of codon Arg<sup>506</sup> (CGA/CGA).

the APC cleavage site at Arg<sup>506</sup>. The occurrence of mutations at Arg<sup>506</sup> was monitored by direct sequencing of the amplified fragment.

Previous studies with bovine factor V have shown that APC partly inactivates factor V by cleavage at the peptide-bond Arg<sup>505</sup>-Gly<sup>506</sup>.<sup>9</sup> Direct sequencing of the corresponding part of factor V cDNA derived from our patients revealed a single G to A transition, which results in substitution of Arg<sup>506</sup> for Gln (figure 1, right panel). 10 of the 27 patients were heterozygous for the Arg<sup>506</sup> to Gln<sup>506</sup> mutation. 8 of the 27 (30%) had an abnormal APC sensitivity ratio ( $\leq 2.0$ ), in agreement with the frequency in similar cohorts.<sup>3,5</sup> The abnormal APC sensitivity ratio was significantly linked to the Arg<sup>506</sup> to Gln mutation (figure 2; U test,  $p < 0.0001$ ). In 3 patients who were heterozygous for the Arg<sup>506</sup> Gln mutation, APC ratio was just above 2.0, and in only 1 patient did an abnormal APC sensitivity ratio (1.9) coincide with the normal Arg<sup>506</sup>/Arg<sup>506</sup> genotype.

Our results indicate that APC resistance in patients with idiopathic thromboembolism was linked to a single mutation at the putative APC cleavage site at Arg<sup>506</sup> in factor V. The data suggest that APC resistance is not due to a deficiency of a cofactor of APC as has been proposed.<sup>3,6,7,10</sup>

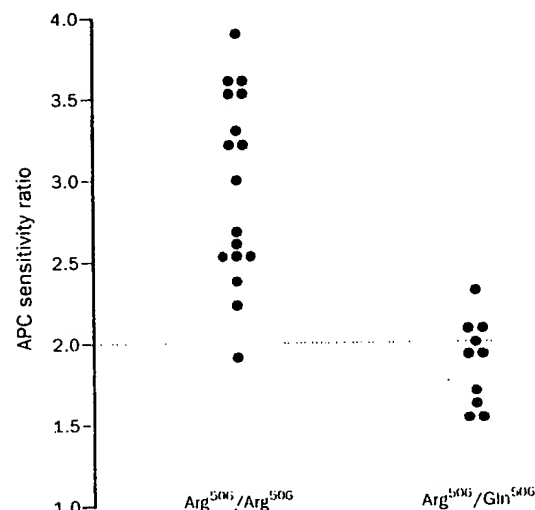


Figure 2: Anticoagulant response to APC

Right = 10 patients heterozygous for Arg<sup>506</sup> → Gln mutation, and left = 17 patients who do not carry the mutation.

but merely reflects the inability of APC to inactivate the pro-coagulant factor V. The high frequency of APC resistance in patients with idiopathic venous thromboembolism suggests that the mutation Arg<sup>506</sup> to Gln may be a major cause of inherited thrombophilia.

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## Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis

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Resistance to activated protein C (APC) is a major cause of familial thrombophilia, and can be corrected by an anticoagulant activity expressed by purified factor V. We investigated linkage between APC resistance and the factor V gene in a large kindred with familial thrombophilia. Restriction fragment length polymorphisms in exon 13 of the factor V gene were informative in 14 family members. The 100% linkage between factor V gene polymorphism and APC resistance strongly suggested a factor V gene mutation as a cause of APC resistance. A point mutation changing Arg<sup>506</sup> in the APC cleavage site to a Gln was found in APC resistant individuals. These results suggest factor V gene mutation to be the most common genetic cause of thrombophilia.

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Heterozygous protein C or protein S deficiency is associated with familial thrombosis,<sup>1</sup> and inherited resistance to activated protein C (APC) as a possible cause of thrombophilia was discovered in a family with thrombosis.<sup>2</sup> It is now well established that APC resistance is a major cause of venous thrombosis,<sup>3-6</sup> and APC resistance in different families appears to be characterised by a molecular similarity.<sup>3</sup> In a cohort of thrombosis patients, APC resistance was at least ten times more frequent than any of the other anticoagulant protein deficiencies (40% vs ≤4%) and in familial thrombophilia, it accounted for more than 50% of cases.<sup>3</sup> APC resistance in the general population is around 5%.<sup>3,5</sup>

We have isolated and characterised the protein that corrects APC resistance, and found it to be identical to factor V.<sup>7</sup> Factor V is pro-coagulant after activation by thrombin, whereas the novel anticoagulant cofactor activity, which we have also found in purified systems (Dr L Shen and BD, Department of Clinical Chemistry, Malmö General Hospital, Sweden), appears to be a property of unactivated factor V. Because APC-resistant plasma contains normal levels of factor V pro-coagulant activity, APC resistance may be caused by mutations in the factor V gene resulting in selective loss of the anticoagulant activity of factor V or in increased resistance to APC of mutant factor Va itself. We have investigated whether APC resistance is due to mutation in the factor V gene in a study of linkage in a large kindred with familial thrombophilia.

The APC resistance test, a modified activated partial thromboplastin time in which the anticoagulant response to standardised addition of APC is measured, was done as described.<sup>2,3</sup> The results were expressed as the APC ratio (clotting time with the APC/CaCl<sub>2</sub> solution divided by clotting time with CaCl<sub>2</sub>). Family members with confirmed APC ratios under 2.0 were considered to be APC resistant.<sup>3</sup> Free and total protein S were measured with a radioimmunoassay.<sup>3</sup> Family members with a concentration of free protein S below the normal limit were considered to be protein S deficient. Their total protein S levels were slightly low or in the low normal range.

Genomic DNA was prepared from EDTA-blood by standard procedures. A sequence of 1188 basepairs (bp) of the factor V gene (nucleotides 2066 to 3254 of the factor V cDNA; sequence from Genebank) was amplified from genomic DNA with two primers 5'GAACCTGGATGTTAACCTCC3' and 5'GGCTTCACTTCTTAGAGGGTG3' (figure). The conditions for polymerase chain reaction (PCR) for 40 cycles of amplification were: 60 s denaturation at 93°C, 30 s annealing at 61°C, and 180 s extension at 72°C. After amplification, the DNA was cleaved with *TaqI* and with *EcoRI*, and subjected to agarose-gel electrophoresis. The region in exon 10 that encodes one of the APC cleavage sites in factor V was PCR amplified from genomic DNA with 5'GGGCTAATAGGACTACTTCTAAATC3' (corresponding to Gly<sup>490</sup>-Ile<sup>497</sup>) and 5'TCTCTTGAAGGAAATGCCCATTA3' (derived from intron sequence provided by Dr W Kane). The PCR conditions were 5 min initial denaturation at 94°C followed by 30 cycles of 60 s denaturation at 93°C, 30 s annealing at 61°C, and 90 s